In silico Design and Characterization of Multi-epitopes Vaccine for SARS-CoV2 from Its Spike Protein

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Abstract

COVID 19 is a disease caused by a novel coronavirus, SARS-CoV2 originated in China most probably of Bat origin. Multi-epitopes vaccine would be useful in eliminating SARS-CoV2 infections as asymptomatic patients are in large numbers. In response to this, we utilized bioinformatic tools to develop an efficient vaccine candidate against SARS-CoV2. The designed vaccine has effective BCR and TCR epitopes screened from the sequence of S-protein of SARS-CoV2. Predicted BCR and TCR epitopes found antigenic, non-toxic and probably non-allergen. Modeled and the refined tertiary structure predicted as valid for further use. Protein–Protein interaction prediction of TLR2/4 and designed vaccine indicates promising binding. The designed multi-epitope vaccine has induced cell-mediated and humoral immunity along with increased interferon-gamma response. Macrophages and dendritic cells were also found to increase upon the vaccine exposure. In silico codon optimization and cloning in expression vector indicates that the vaccine can be efficiently expressed in *E. coli*. In conclusion, the predicted vaccine is a good antigen, probable no allergen, and has the potential to induce cellular and humoral immunity.

Keywords COVID19 · SARS-CoV2 · B cell epitopes · T cell epitopes · Vaccine · Immune-simulation

Introduction

In December 2019, a group of patients from Wuhan city of China was found to have pneumonia-like symptoms and diagnosed with the infection of beta coronavirus. Soon the infection spread across China and later spread all over the globe. These infections were named COVID 19 disease and the virus as SARS-CoV2 by WHO (Sohrabi et al. 2020). On 30 January 2020 due to its spread in more than 120 countries, WHO declared the COVID 19 pandemic as a public health emergency of international concern. The Novelty of SARS-CoV2 is its rapid spread may be due to asymptomatic patients (Gandhi et al. 2020; Bi et al. 2020) and highly sophisticated, time-consuming diagnostic methods (Chan et al. 2020; Yelin et al. 2020, Li and Xia 2020). In the report published by WHO on the spread of COVID 19 infection, 4 million new cases were emerged with a cumulative number of 244 million cases worldwide and 4.6 million deaths since from COVID 19 pandemic declaration (WHO 2020).

In India, due to lockdown positive cases are in control and spread is also marginal. However, still after two years from the pandemic declaration, SAR CoV 2 succumbing cases are increasing due emergence of new more transmissible variants of SARS-CoV2. Till September 16, 2021, a total of 33.35 million cumulative cases with 443,928 deaths were reported by the ministry of health and family welfare, Govt. of India (<https://www.mohfw.gov.in/>). To date, no promising antiviral drug is available to combat SARS-CoV2 infections. There are few drug candidates in clinical trials, but the process of drug development is slow and time-consuming (Mitjà and Clotet 2020). In South Korean clinical practices, Lopinavir/Ritonavir combination was found significantly effective in lowering the viral load to no detectable or little SARS-CoV2 titer (Wen et al. 2020). But another research group showed that the same combination was ineffective beyond standard care (Cao et al. 2020). Another drug pair, hydroxychloroquine, an antimalarial drug, and azithromycin reported having an association in the reduction of viral load (Gautret et al. 2020a, b). Nevertheless, QT interval prolongation may cause life-threatening arrhythmia (Chorin et al. 2020). In a large scale study both drugs and drugs alone compared with neither drug was associated with mortality (Rosenberg et al. 2020; Molina et al. 2020). In a drug repurposing study,

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remdesivir, lopinavir, emetine, and homoharringtonine have significantly inhibited the replication of SAR-CoV2 (Choy et al. 2020). But this was in vitro study, and preliminary clinical data suggest no relation with mortality. Moreover, a multicentric study conducted for remdesivir showed no role in lowering the viral load and failed to provide clinical benefits in the patients with Severe COVID (Wang et al. 2020). Prevention is a better option than treatments to break the chain of SARS-CoV2 infections. Several shreds of evidence suggest and support the importance of vaccines to eliminate the COVID19 pandemic (Chen et al. 2020; Bai et al. 2020; Prem et al. 2020). Various epidemiological surveys proved that naturally acquired immunity could decrease the SARS-CoV2 titer (Shi et al. 2020; Prompetchara et al. 2020). More than 80% of patients develop mild and 14% develop severe symptoms of COVID19 with zero case fatality rate (Spychalski et al. 2020). Data published on efficacy of various vaccines released in the market have shown promising hope in the controlling spread of SAR-CoV2 infections (Polack et al. 2020; Oliaro et al. 2021). However, the evolution of new SAR-CoV2 variants like delta and omicron can evade the immunity and lower effectiveness of the vaccines (Lopez Bernal et al. 2021; Planas et al. 2021). On these circumstances, it is indispensable to develop a vaccine that will be effective against various variants of SAR-CoV2..

A highly effective vaccine must have common and more than one immunogenic region from the strains that will raise the various immunological attacks against different variants of a virus. The bioinformatics approach can accelerate the process by predicting common potential candidate peptides. Such peptides can be tailored together with adjuvant protein to enhance the immunological response. Vaccines against MERS, Ebola, and human papillomavirus were designed and successfully developed using bioinformatics approaches (Shey et al. 2019).

SARS-CoV2 belongs to the beta-coronaviridae family including four endemic viral pathogens viz HCoV-HKU1, HCoV-OC43, HCoV-229E, HCoV-NL63, two epidemic viruses like the Middle East Respiratory Syndrome virus (MERS), and SARS (Osman et al. 2020). This virus is a non-segmented positive-strand RNA virus with an envelope. Envelop proteins are categorized into structural, non-structural, and accessory proteins. Structural proteins are involved in protection and bind to the host. Several proteins of SAR-CoV2 are essential for virulence and pathogenesis. For example, Nucleocapsid (N) protein N, is essential for RNA binding and its replication and transcription (Snijder et al. 2016). Envelope (E) and membrane (M) proteins are instrumental in virus assembly and virulence promotion (Ruch and Machamer 2012; Alsaadi et al. 2019). In addition, E and M proteins effectively induce an immune response (Ahmed et al. 2020). Spike (S) protein, a structural protein is responsible for binding to a receptor

on the host cell called as Angiotensin-converting enzyme 2 (ACE2) (Hoffmann et al. 2020). S-Protein undergoes proteolytic cleavage by TMPRSS2 that allows subsequent entry of the virus into cells through endocytosis (Glowacka et al. 2011). S- protein was also found to be involved in the activation of T cell response (Li et al. 2020; Grifoni et al. 2020). Entire S protein expressed in chimpanzee adeno 38 (ChAd)-vector showed protection against SARS-CoV2 in mice and *rhesus* macaques (van Doremalen et al. 2020). Single-dose of ChAdOx1 nCoV-19 vaccine is sufficient to elicit a humoral and cell-mediated immune response in both the animals. Viral load also was found to be reduced compared to control animals and symptoms of pneumonia were absent. Here, in this communication a multi-epitopes vaccine is designed from S Protein of SAR-CoV2. This vaccine has ideal properties like good stability at room temperature, immunogenic, antigenic and non-allergen in nature. All the epitopes are effective in the stimulation of humeral as well and cell-mediated immunity.

Methods

Selection of Protein for Epitope Prediction

Complete Spike protein sequence (P0DTC2) of SARS-CoV2 was downloaded in fasta format from UniProt protein database. This sequence was used for further analysis and obtaining potential epitopes for B and T cell receptors.

T Cell Epitope Prediction

Various tools were used to predict the epitopes presented to T cell receptors. Potential epitopes of various bacteria and viruses were predicted by using such online epitopes predicting tools. The following tools were used for the prediction of TCR (T cell Receptor) epitopes IEDB MHC-I processing predictions, MHC-NP, netCTLpan1.1, RANK-PEP, and netMHCpan4.0. All these tools were used to screen the potential peptides as epitopes for TCR. These tools have various methods to predict epitopes. IEDB (Instructor/Evaluator Database) is a web-based epitope analysis resource that includes tools for T cell epitope prediction, B cell epitope prediction, and other analysis tools like epitope conservancy, etc. (Fleri et al. 2017; Dhanda et al. 2019). This resource uses methods based on artificial neural network (ANN), stabilized matrix method (SMM), and Combinatorial Peptide Libraries (CombLib), predict the peptides' way that processed naturally and presented by MHC I (Calis et al. 2013).

Analysis of Immunological Properties

The properties like immunogenicity, toxicity, allergen, and antigenicity were analyzed to predicted the TCR epitopes. IEDB MHC class I immunogenicity server and conservancy tool were used for the determination of immunogenicity and conservancy. The immunogenicity of a peptide MHC complex (<http://tools.immuneepitope.org/immunogenicity/>) was assessed keeping all the parameters at default. Protein sequence variants used setting sequence identity 100% and other parameters default (Calis et al. 2013). ToxiPred (<http://www.imtech.res.in/raghava/toxinpred/index.html>) a online tool predicted the toxicity considering the physicochemical properties of selected peptides (Mishra et al. 2014). Online server AllergenFP v.1.0 (<http://ddgpharmfac.net/AllergenFP/>) was used to predict peptides as allergens (Dimitrov et al. 2014a, b). The epitopes' antigenicity was analyzed by online server VaxiJen v2.0 (<http://www.ddg-harmfac.net/vaxijen/VaxiJen/VaxiJen.html>) (Doytchinova and Flower 2007b). The threshold value was set to 0.5. It is alignment- independent predictor based on auto-cross covariance (ACC) transformation epitopes sequences into uniform vectors of principle amino acid properties. The accuracy of this server varies between 70% and 89% depending on the targeted organism.

Linear B Cell Receptor Epitope Prediction

Six different methods were used for the prediction of B cell receptor (BCR) epitopes. All these methods generate fragments of the protein. To predict the B cell epitopes, it is necessary to find a linear sequence of B cell epitopes in the protein sequence. BepiPred linear epitope prediction server (<http://www.cbs.dtu.dk/services/BepiPred/>) uses a hidden Markov model and propensity scale method (Jespersen et al. 2017). Similarly, other properties were also being considered to predict good B cell epitopes. Different methods calculate various properties at the IEDB server (<http://tools.iedb.org/bcell/>). Kolaskar–Tongaonkar antigenicity scale provides physiology of the amino acid residues (Kolaskar and Tongaonkar 1990). Emini Surface accessible score for the accessible surface of the epitope (Emini et al. 1985). Secondary structure of epitopes also has a role in antigenicity. Karplus–Schulz flexibility score (Karplus and Schulz 1985) and Chou–Fasman β turn methods were used for flexibility and β turns prediction (Garnier 1978) respectively. Parker hydrophilicity prediction method was used for the determination of in silico hydrophilicity of peptides (Parker et al. 1986).

Engineering of Multi-epitope Vaccine Sequence

High-scored and common peptides predicted by various tools were selected for the deriving sequence of the potential vaccine candidate. GP GPG and AAY linked together with selected epitopes for T and B cell receptors. For enhanced immunogenicity, OmpA (GenBank: AFS89615.1) protein was chosen as an adjuvant and was linked through EAAAK at the N-terminal site of the vaccine.

Prediction of Immunogenic Properties of Designed Vaccine

The antigenicity of the chimeric vaccine was predicted by VaxiJen v2.0 (<http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html>) and ANTIGENpro online tools. VaxiJen 2.0 is an alignment-free antigenicity prediction tool that utilizes auto cross-covariance transformation of protein sequence into uniform vector vectors of principal amino acid properties (Doytchinova and Flower 2007b). ANTIGENpro (<http://scratch.proteomics.ics.uci.edu/>) is also an online tool that utilizes a protein microarray dataset to predict the antigenicity (Cheng et al. 2005). Based on the cross-validation experiment accuracy of this server is estimated to be around 76% using a combined dataset. AllerTop v2.0 and AllergenFP were two online tools utilized for the prediction of allergenicity of chimeric protein. Amino acid E-descriptors, auto- and cross-covariance transformation, and the k nearest neighbors (kNN) machine learning methods are the basis of AllerTop v2.0 (<http://www.ddg-pharmfac.net/AllerTOP/>) for allergenicity prediction (Dimitrov et al. 2014a). AllergenFP is a descriptor-based fingerprint, alignment-free tool for allergenicity prediction (Dimitrov et al. 2014a, b). The tool uses a four-step algorithm. In the first step, proteins were analyzed based on their properties like hydrophobicity, size, secondary structures formation, and relative abundance. In the second step, generated strings were converted into vectors of equal length by ACC. Then, in the third step, vectors were converted into binary fingerprints and compared in terms of the Tanimoto coefficient. In last step, applying this approach to known allergen and non-allergen can identify 88% of allergen/non-allergen with Mathew's Correlation coefficient of 0.759.

Prediction of Solubility and Physicochemical Properties

For Solubility prediction of multi-epitopes chimeric vaccine, PROSO II server (<http://mbiljj45.bio.med.uni-muenchen.de:8888/prosoII/prosoII.seam>) was utilized. PROSO II server works on an approach of the classifier which utilizes the difference between TargetDB and PDB and insoluble proteins of TargetDB (Smialowski et al. 2012). The accuracy of this server is 71% at the default threshold of 0.6. Prototparam, the online webserver, was exploited for the evaluation of

physicochemical properties. The properties like amino acid composition, theoretical pI, instability index, in vitro and in vivo half-life, aliphatic index, molecular weight, and grand average of hydropathicity (GRAVY) were evaluated.

Secondary and Tertiary Structure Prediction

PSIPRED and RaptorX Property online servers were used to determine the secondary structure of the predicted vaccine. PSIPRED is a publicly available web server, includes two feed-forward neural networks works on output obtained from PSI-BLAST (McGuffin et al. 2000). PSIPRED 3.2 attains an average Q3 score of 81.6% obtained using very stringent cross approval strategies to assess its performance. RaptorX property is another web-based server prediction secondary structure of protein without template (Wang, et al. 2016a, b). This server utilizes DeepCNF (Deep Convolutional Neural Fields), a new machine learning model that simultaneously predicts secondary structure and solvent accessibility and disorder regions (Wang, et al. 2016a, b). It accomplishes Q3 score of approximately 84 for 3 state secondary structures and approximately 66% for three state solvent accessibility.

The tertiary structure of multi-epitopes chimeric vaccine candidate was built using I-TASSER online server (<https://zhanglab.ccmb.med.umich.edu/I-TASSER/>). The I-TASSER (Iterative Threading Assembly Refinement) webserver utilizes the sequence-to-structure-to-function paradigm to build protein structure (Roy et al. 2010). It is a top-ranked 3D protein structure web server in community-wide CASP experiments (Zheng et al. 2019).

Tertiary Structure Refinement and Validation

Two web bases servers were used to refine the 3D structure of the multi-epitopes chimeric protein. Initially, the Modrefiner server (<https://zhanglab.ccmb.med.umich.edu/ModRefiner/>) and finally GalaxyRefine server (<http://galaxy.seoklab.org/cgi-bin/submit.cgi?type=REFINE>) was used. Modrefiner server is based on an algorithm for atomic-level structure refinement that utilizes α trace, main chain, or atomic model (Xu and Zhang 2011). The output structure was refined in terms of the accurate position of the side chains hydrogen bond network and fewer atomic overlaps. On the other hand, the Galaxy server rebuilds the 3D structure, performs repacking, and uses molecular dynamic simulations to accomplish overall protein structure relaxation. Structure refined by the Galaxy server is of the best quality as per community-wide CASP10 experiments (Ko et al. 2012).

ProSA-web (<https://prosa.services.came.sbg.ac.at/prosa.php>), The ERRAT server (<http://services.mbi.ucla.edu/ERRAT/>) and RAMPAGE (<http://mordred.bioc.cam.ac.uk/~rapper/rampage.php>) web servers were utilized for 3D

structure validation obtained after galaxy server refinement. ProSA web server calculates the quality score as Z score that should fall in a characteristic range (Wiederstein and Sippl 2007). Z score obtained for a specific input protein is in context with protein structure available in the public domain. ERRAT server analyses non-bonded atom–atom interaction with the refined 3D structure of protein compared to high resolved crystallographic protein structures (Colovos and Yeates 1993). Ramachandran plot displays energetically allowed and disallowed dihedral psi and phi angles of amino acids. The plot is calculated based on van der Waal radius of the protein side chain. RAMPAGE server determines the Ramachandran plot for a protein that includes the percent of residues in allowed and disallowed regions (Lovell et al. 2003).

Discontinuous B Cell Epitope Prediction

More than 90% B cell epitopes are discontinuous that is they are present in small segments on linear protein and brought to proximity during protein folding. Discontinuous B cell epitopes for the designed vaccine were predicted by ElliPro online tool (<http://tools.immuneepitope.org/tools/ElliPro>) at IEDB. In this tool, three algorithms were implemented to determine the discontinuous B cell epitopes. 3D structure of the input protein is approximated as the number of ellipsoid shapes, calculate protrusion index (PI) and clusters neighboring residues. ElliPro defines the PI score of each residue based on the center of mass of residue residing outside the largest possible ellipsoid (Ponomarenko et al. 2008). considering other epitope predicting tools ElliPro gave an AUC value of 0.732, best of all (Ponomarenko et al. 2007).

Protein–Protein Docking of Designed Vaccine with TLR2 and TLR4

Molecular docking server HADDOCK (<https://bianca.science.uu.nl/haddock2.4/>) was used to see the interaction of the designed vaccine and TLR4. HADDOCK (High Ambiguity Driven protein–protein DOCKing) is an information-derived flexible docking server (Van Zundert et al. 2016). Galaxyrefine 3D model of multi-epitopes chimeric proteins, adjuvant TLR2 (PDB ID: 6NIG), and TLR4 (PDB ID: 4G8A) were uploaded for docking at HADDOCK server, keeping all the parameter default. Finally, the top five models were downloaded and by PRODIGY (PROtein binDIng enerGY prediction) webserver (<https://nestor.science.uu.nl/prodigy/>) was utilized for prediction of binding affinities (Xue et al. 2016).

Immune Simulation

In silico cloning of the vaccine was predicted by the C-ImmSim server (<http://150.146.2.1/C-IMMSIM/index>).

php). C-immSim Server is a freely available web-based server that utilizes a position-specific scoring matrix (PSSM) for the prediction of immune epitopes and machine learning techniques for immune interaction (Rapin et al. 2010; Rapin et al. 2011).

JCat (Java Codon Adaptation Tool) server was utilized for reverse translation and codon optimization. Codon optimization is carried out to express the construct in the *E. coli* host. JCat (<http://www.prodocor.de/JCat>) output display sequence of nucleotide, other important properties of sequence that includes codon adaptation Index (CAI), percent GC content essential for to assess the protein expression in the host (Grote et al. 2005). Finally, the vaccine construct was cloned in the pET30a (+) plasmid vector by adding XhoI and XbaI restriction sites at C and N terminus, respectively. The Snapgene tool was used to clone the construct to ensure cloning and expression (Goldberg et al. 2018).

Results

Prediction of B and T Cell Receptor Epitopes

T Cell Receptor Epitopes

IEDB recommended MHC-NP, netCTLpan1.1, RANKPEP, and netMHCpan3.0 servers predicted the potential candidate epitopes from the spike glycoprotein sequence of SARS-CoV2. Epitopes commonly predicted by at least four prediction tools were selected for further analysis parameters. Four such epitopes with a high score were predicted by four different tools (Table 1A). All the four epitopes were immunogenic and antigenic, conserved 100%, and predicted to be non-allergen (Table 1B). Immunogenicity scores for the predicted epitopes range from 0.3858 to 0.0961. All the epitopes were with positive scores hence considered for

further analysis. All the peptides were predicted to be non-toxic by toxipred online toxicity prediction tools.

B Cell Receptor Linear Epitopes

After provision of spike protein sequence to the BepiPred server showed an average score of 0.407, with 0.695 maximum and 0.163 minimum scores (Fig. 1). Other properties like the physiology of amino acid residues, accessible surface, hydrophilicity, Flexibility, and β turns were also determined (Table 2 and Fig. 1). Peptides obtained in six different methods were arranged according to higher to lower scores and 2% of high score peptides were selected for overlap screening. Regions commonly overlap in at least four prediction methods selected as potential B epitopes. Regions 250 to 261 and 1246 to 1267 were overlapped regions as per the condition applied. Region 250 to 260 includes BepiPred region 250–260, 251 to 257 predicted by Karplus-Schulz flexibility and Chau-Fasman β turn prediction methods, and 250 to 257 regions predicted by parker hydrophilicity prediction method. Similarly, region 1246 to 1267 covers region predicted by BepiPred 2.0 (1252–1267), Emini surface accessible (1257–1262), Kolaskar, and Tongoankar method (1247–1254), Chau-Fasman β turn prediction method (1246–1252) and Parker hydrophilicity method (1256–1262).

Engineering of Multi-epitope Vaccine Sequence

Three peptides with high scores predicted for T cell receptors, and two peptides for B cell receptors were ligated together by GPGPG or AAY. Additionally, OmpA (GenBank: AFS89615.1) was linked at the N-terminal end of the designed polypeptides by EAAAK linker as an adjuvant protein. For purification purposes, six histidine residues were added. The final amino acid residues of the designed vaccine

Table 1 **A** Common peptides predicted by at least four TCR epitopes prediction tools; **B** Immunogenic properties of that peptides. In the case of immunogenic properties, peptide number 3 has a negative score for antigenicity hence neglected for further analysis

(A)							
Epitopes	Sequence	Amino acid number	Net CTLpan	RankPep	IEDB	MHC NP	Net MHC 4.0
TCR1	WYIWLGFIAGLIAI	1214–1227	0.8	42.06	0.32	0.2815	0.3
TCR2	FVSNNGTHWV	1095–1105	0.3	–	0.07	0.344184	0.17
TCR3	KLPDDFTGCV	423–432	0.4	39.12	–0.68	0.591582	0.3
TCR4	KLNDLCFTNV	385–393	0.4	34.15	0.13	0.710803	0.9
(B)							
Epitopes	Sequence	Immunogenicity	Conservancy	Toxicity	Antigenicity	Allergen	
TCR1	WYIWLGFIAGLIAI	0.38584	100	Non-toxic	0.5798	Probable Non-allergen	
TCR2	FVSNNGTHWV	0.18796	100	Non-toxic	0.5982	Probable Non-allergen	
TCR3	KLPDDFTGCV	0.1001	100	Non-toxic	–0.5308	Probable Non-allergen	
TCR4	KLNDLCFTNV	0.0961	100	Non-toxic	2.6927	Probable Non-allergen

Fig. 1 Graphical representation of prediction of B cell epitopes Immunogenic peptide predicted by various tools probable epitopes are scored above the threshold, showed here as yellow. **A** Parker

hydrophilicity prediction, **B** Chau-Fasman β turn prediction **C** Karplus-Schulz flexibility, **D** Emini Surface accessible, **E** Kolaskar and Tongaonkar, **F**: BepiPred Linear Epitope Prediction 2.0

Table 2 B cell receptor epitope prediction, **A** Common B cell receptor epitopes predicted by various methods **B** Score obtained B cell receptor epitopes for Spike glycoprotein of SARS-CoV2

(A)			
Method	Average score	Maximum score	Minimum score
BepiPred 2.0	0.470	0.183	0.695
Emini Surface accessible	1.0	0.042	6.051
Chua-Fasman β turn	0.997	0.541	1.484
Karplus-Schulz flexibility	0.993	0.876	1.125
Kolaskar–Tongaonkar antigenicity	1.041	0.866	1.261
Parker hydrophilicity	1.238	−7.629	7.743
(B)			
BCR epitopes	Region	Sequence	
BCR1	250–260 (11 aa)	TPGDSSSGWTA	
BCR2	1246–1267 (22 aa)	GCCSCGSCCK-FDEDDSEPVLKG	

were 454 when all the five peptides, linkers, and adjuvant were ligated (Fig. 2).

Prediction of Immunogenic Properties of the Designed Vaccine

The antigenicity of the designed multi-epitopes vaccine predicted by the VaxiJen v2.0 server was 0.7048 with probable antigen annotation for the virus as a target organism at 0.5 thresholds. Antigenicity predicted for the same sequence

Fig. 2 Schematic presentation of the final multi-epitope vaccine peptide. The 599-amino acid long peptide sequence containing an adjuvant (Red) at the amino-terminal end linked with the multi-epitope sequence through an EAAAK linker (black). BCR epitopes (yellow) are linked using GPGPG linkers (cyan) while the TCR epitopes (blue) are linked with the help of AAY linkers (green). A 6x-His tag is added at the Carboxy terminus for purification and identification purposes (Color figure online)

Table 3 Analysis of the physicochemical and immunological properties of the designed vaccine for SARS-CoV2

Characters	score
Antigenicity (AntigenPro and VaxiJen v2.0)	0.765946 and 0.7048 (Probable antigen)
Allergen (AllergenFP and AllerTop)	0.84 and 0.77 (probable non-allergen)
Possible solubility	0.9557226
Number of amino acids	454
Molecular weight	48.49368
pI	7.94
The instability index (II)	24.58
Aliphatic index	79.02
Grand average of hydropathicity (GRAVY)	-0.172

of vaccines by the AntigenPro server was 0.765946. At the same time 0.953508 was predicted as probable solubility upon overexpression. The antigenicity of peptides without adjuvant sequence was 0.671028 with 0.861451 probable solubilities of peptides upon overexpression. The results obtained indicate that with and without adjuvant predicted vaccine sequence is potentially antigenic. AllergenFP and AllerTop servers predicted that both vaccines with adjuvant and without adjuvant are probable non-allergen with 0.84 and 0.77 Tanimoto similarity indexes, respectively.

Prediction Physicochemical Properties

The computed molecular weight of the designed vaccine was 48.49368 kDa and the theoretical pI was 7.96. Predicted pI indicates the slight alkaline nature of the vaccine (Table 3). The estimated half-lives were 30 h in mammalian reticulocytes (in vitro), >20 h in yeast (in vivo), and >10 h in *Escherichia coli* (in vivo). The vaccine was highly stable with an instability index of 24.58 classifying the protein as stable. The estimated aliphatic index was 79.02 indicating high thermo-stability of the protein (Doytchinova and Flower 2007a). The predicted GRAVY score was -0.175 (Table 3). The negative GRAVY score indicates that protein is hydrophilic and will interact with water molecules (Dimitrov et al. 2014b).

Secondary Structure Prediction

Secondary structure predicted by online tool RaptorX property includes 16% alpha-helix region, 42% beta-sheet region, and 40% coil region. Furthermore, solvent accessibility of amino acid residues was predicted to be 42% exposed, 27% medium exposed and 24% buried. A total of 56 residues (12%) were predicted as residues in the disordered region. Pictorial representation of secondary structure predicted of final protein by PSIPRED is shown in Fig. 3.

Tertiary Structure Prediction Refinement and Validation

I-TASSER predicted the top five 3D structure models of designed vaccines utilizing 10 threading templates. Z scores for these 10 templates were in the range of 0.95 to 5.50, indicating good alignment with the sequence submitted. C score, critical in the quality of the built model which was quantitatively measured. C score was in the range of -5 to 2 signifies the best model quality. C score of the top five models ranging from -4.14 to -3.41. A model with a high C score i.e. -3.41, estimated TM score 0.34 ± 0.11 and estimated RMSD 15.6 ± 3.3 was chosen for further analysis (Fig. 4A) (Smialowski et al. 2012).

For refinement purposes, the tertiary structure predicted by I-TASSER is initially submitted to ModRefiner and finally to GalaxyRefine. Among the top five refined models, model 5 was found to be the best model based on various parameters like GDT-HA = 0.8398, RMSD = 0.696, MolProbity = 3.526, and Rama favored = 77.1 (Fig. 4B).

The Ramachandran plot at RAMPAGE webserver validated the quality of refined model 5. 79.5% of modeled vaccine residues were in the favored region, 13.1% in the allowed region and 7.3% were in the outlier region (Fig. 4D). Error in the model is analyzed by the ProSA web and ERRAT web server. Z score was -1.64 (Fig. 4E) and the

Fig. 3 Graphical representation of secondary structure characters; 42% beta-sheet region, 40% coil region and 16% alpha-helix region

ERRAT score index was 74.387 (Fig. 4C). Ramachandran Plot Z score and ERRAT score showed that refined model 5 is of good quality and can be used further.

Dis-continuous Epitopes for B Cell

Four discontinuous epitopes, including 265 residues, were predicted from the sequence of Model 5. The score of the predicted epitopes ranges from 0.58 to 0.749. The shortest

Fig. 4 Vaccine tertiary structure modeling, refinement, and validation **A** Tertiary structure of ITASSER modeled multi-epitopes Vaccine along with OmpA as adjuvant; **B** Refined 3D model (green) after GalaxyRefine superimposed on ITASSER crude model (violet)

C Errata score for a refined model with graphical representation, **D** Ramachandran plot for a refined 3D model of vaccine, **E** Z-score – 1.64 by ProSA webtool (Color figure online)

and longest discontinuous B cell epitopes are 14 and 97 residues long respectively.

Molecular Docking

HADDOCK web bases server was utilized to dock the designed vaccine to TLR2 and TLR 4. TLR2/4 eliciting immune response toward the designed vaccine was analyzed by conformational change concerning adjuvant TLR2/4 complexes. Top models from each group with the lowest HADDOCK score were selected. HADDOCK score for TLR2/Adjuvant and TLR2/Vaccine were –89.3 and –95.9 kcal/mol, respectively. Also, relative binding energies were –9.8 and –12.8 for the TLR2/adjuvant and TLR2/vaccine, respectively. Similarly, HADDOCK scores for the TLR4/adjuvant complex and TLR4/vaccine complex were –75.6 and –139.9 kcal/mol, respectively. The relative binding energy of the TLR4/adjuvant complex was –10.7 kcal/mol and –8.2 for the TLR4 vaccine complex. The difference in scores and binding energies of adjuvants and vaccines indicates the change in conformation that may stimulate TLR2/4 receptors. Even there was a difference in the number of interacting residues at the juncture. TLR2/Adjuvant complex have charged-charged 2, charged

polar 6, charged-apolar 24, polar-polar 2, polar-apolar 22 and apolar-apolar 21. While TLR2/Vaccine has charged-charged 3, charged polar 7, charged-apolar 15, polar-polar 1, polar-apolar 13, and apolar-apolar 119 (Fig. 5A, B). Similarly, TLR4/Adjuvant complex have charged-charged 3, charged polar 10, charged-apolar 21, polar-polar 3, polar-apolar 15 and apolar-apolar 20. While TLR4/vaccine complex has charged-charged 1, charged-polar 9, charged-apolar 10, polar-polar 9, polar-apolar 15 and apolar-apolar 8 interface contacts were observed (Fig. 5C, D).

Codon Optimization

Enhance expression of nucleotide construct in *Escherichia coli*, is reverse translated in Jcat online web tool. The total number of nucleotides in the construct was 1350. After codon optimization, CAI was 1.0 and the average GC content of the optimized nucleotide construct was 50.7340 indicating good probable expression of the vaccine in *E. coli* K12. To clone the gene XhoI and XbaI restriction sites are added at N and C terminus of the construct respectively and was cloned in pET30a + plasmid, by using SnapGene software (Fig. 6).

Fig. 5 Haddock docking cartoon models showing interaction **A** TLR2-Vaccine; TLR2 in red and Vaccine in blue; **B** TLR2-Adjuvant; TLR2 in red and Adjuvant in blue; **C** TLR4-Vaccine; TLR4 in red and Vaccine in red; **D** TLR4-Adjuvant; TLR4 in red and Adjuvant in blue

Immune Simulation

C-ImmSim Immune simulator webserver was used for determining the ability of a vaccine to induce immunity. Results obtained are consistent with the experimental results published elsewhere. Within the first week of vaccine administration, the primary response observed was the high-level secretion of IgM. The secondary and tertiary immune response seen with an increase in the B cell number, level of IgM, IgG1 + IgG2, and IgM + IgG with decreasing concentration of the antigen (Fig. 7A). Different B cell isotype populations were also found increasing indicating isotype switching and memory formation (Fig. 7B). B cell activities were also found high, especially B Isotype IgM and IgG1,

which were observed with prominent memory cell formation (Fig. 7B). Similarly, the cell populations of Th and Tc cells are found high along with memory development (Fig. 7C, D). Also, active macrophages were found to be increased consistently after each antigen shot and declined upon antigen clearance (Fig. 7E). Another cell from cell-mediated immunity, dendritic cells were also found increased (Fig. 7F). IFN γ and IL2 expression were found high with low Simpson Index indicating sufficient immunoglobulin production, suggesting good humoral immune response (Fig. 7G). Simpson Index (D) is a measure of diversity. An increase in D over time indicates the emergence of different epitope-specific dominant clones of T-cells. The smaller the D value, the lower the diversity.

Fig. 6 In silico cloning of designed vaccine construct in pET30a+ expression vector where the yellow arc segment represents cloned construct and the remaining segment represents vector backbone

Discussion

To date there is no sure cure available for COVID19, suggesting the urgent requirement of drug or vaccine to control the spread of SAR-CoV2 infections. Advancements in bioinformatics tools, the vaccine development process can be facilitated by identifying potential epitopes for T and B cells that will lead to a vaccine for the prevention of COVID19. Several reports and animal trials suggest that S-protein can be a potential target for vaccine development (Prompetchara et al. 2020; Tai et al. 2020; Du et al. 2009; Pogrebnyak et al. 2005; Jiang et al. 2005; Hotez et al. 2020). Several reports are suggesting the importance of Spike protein in the activation of cells of immunity and vaccine development (Tufan et al. 2020; Prompetchara et al. 2020; Zhang et al. 2020a, b). HLA-A2 restricted epitopes from S protein of SARS-CoV2, elicit T cell-specific immune response in SARS-CoV2 recovered patients (Wang et al. 2004). Similarly, serum samples of SARS-CoV2 recovered patients were sufficient to neutralize epitope-rich regions on the spike S2 protein from SARS-CoV2 (Zhong et al. 2005). Programming

Fig. 7 Immune response simulation by a designed vaccine, **A** Immune response upon antigen exposure; **B** B cell population per state (cells/mm³); **C**: TH cell population state (cells/mm³); **D** TC cell population state (cells/mm³); **E** Activity of macrophage population in three subsequent immune responses; **F** Dendritic cell population state

(cells/mm³) upon antigen exposure; **G**: Cytokine-induced by three injections of vaccine after a one-week interval. The main plot shows cytokine levels after the injections. The insert plot shows IL-2 level with the Simpson index, **D** indicated by the dotted line

of a live attenuated form of the pathogen is a commonly used strategy for vaccine development. Despite the efficacy of such vaccines, the reversion of virulence remains a challenge and is not utilized in weak immune patients (Plitnick and Herzyk 2013; Plitnick 2013). Here in case SARS-CoV2 due to its high communal transmission vaccine research requires highly skilled workers, sophisticated instrumentation, and biosafety level III facility. This may likely slow down the process of vaccine development, enhancing cost that leads to a restriction on the availability of vaccines for the mass population. Advancements in the field of bioinformatics and molecular biology techniques have provided an opportunity for the development of a vaccine with high efficacy that reduces vaccine development time, and cost-effective products that may reduce the cost of vaccine in time for the large population. Various research groups across the world are searching for highly effective vaccine to stop the spread of virus. Research communications published till now has one common thing and that is spike protein as the protein of interest for the development of vaccine. Covisheid of Oxford, JNJ-78436735 of Janssen Pharmaceuticals and Gam-COVID-Vac of Sputnik are actually contained whole spike protein as active ingredient of SARS-CoV2 origin. On the other hand, Moderna's mRNA vaccine codes spike protein to provide the immunity against SARS-CoV2. As already described above the advantages of bioinformatic

tools in vaccines development process, there are some research papers in the public domain focusing on the multi-epitopic vaccine candidate against SARS-CoV2. Spike protein is at the center for most of the studies and used various strategies. Various structural proteins like nucleocapsid ORF3a, envelop and membrane protein were evaluated for epitope screening including spike proteins (Kumar et al. 2020; Rahman et al. 2020; Enayatkhani et al. 2021). Long stretches (more than 20 aa), short stretches (less than 20 aa) along various linker proteins were the primary constituent of most of the multi-epitopic SARS-CoV2 vaccine (Kumar et al. 2020; Abraham Peele et al. 2021). Recently deep learning approach was used to search the epitopes out of spike protein sequence for the prediction of vaccine subunit. DeepVacPred a AI-based frame work predicted 26 vaccine subunits within a second, skipping almost 95% unnecessary prediction and manual curing (Yang et al. 2021).

In this study, we designed a multi-epitopes vaccine construct from the S-protein of SARS-CoV2. Several online tools were utilized for the designing of epitopes and thereby a vaccine. From various epitopes predicted by the online server based on common sequence and high score, three TCR and two BCR epitopes were selected as part of COVID19 vaccine. All the five TCR and BCR epitopes were linked by GPGPG and AAY linker peptides (Fig. 2). To enhance the antigenicity of TCR/BCR linked epitopes

peptide, adjuvant OmpA were linked by EAAAK linker sequence for a complete vaccine design which was found to restore antigenicity and non-allergen status. (Table 2). For a successful vaccine candidate, secondary and tertiary structures characters play an essential role. The secondary structure of the designed vaccine contains 16% alpha helixes, 42% beta-sheets and 40% coils. Upon refinement tertiary structure of the designed vaccine showed improvement to a desirable level as indicated by the Ramachandran plot. The designed vaccine showed a high antigenicity score, no probable allergen.

TLR are essential receptor proteins in the activation of the innate immune response. That recognizes and responds by induction of immune reactions to PAMPs (pathogen-associated molecular patterns). Various TLRs have been shown to activate the immune response to the virus through their interaction with nucleic acids and envelopes proteins. TLR3 and TLR7/8 are involved in nucleic acid detection, while TLR2 and TLR4 recognize envelope glycoproteins (Xagorari and Chlichlia 2008). Interaction of S protein with TLR2 increases the production of IL8 in human monocyte macrophages (Dosch et al. 2009). TLR 2 is also involved in eliciting innate immune response upon recognition of several other virus components (Finberg and Kurt-Jones 2004; Luo et al. 2020; Aguilar-Briseño et al. 2020; Lin et al. 2020). In a comparative binding efficacy of S-proteins to TLRs, TLR 4 showed the strongest protein–protein interaction with hydrogen and hydrophobic bonds on the extracellular domain (Choudhury and Suprabhat 2020). In molecular docking analysis, TLR2 and TLR4 showed stable protein–protein interaction with the designed vaccine compared to adjuvant protein. TLR 2 showed a more stable interaction than TLR4, which is consistent with the previous report (Choudhury and Suprabhat 2020). Stable interaction of designed vaccine indicates the efficiency of vaccine for activation of TLRs, involved in dendritic cell activation, thereby subsequent antigen processing, and presentation on the surface to T cells (Englmeier 2020; Li et al. 2020). The immune stimulation showed typical natural immune response patterns upon multiple exposures to the same antigen. The server predicted elevated levels of B cell and T cell for a longer time on repeated exposure of antigen. Increased levels of antiviral cytokine IFN γ and IL2 indicate the potential of subsequent activation of T-helper cells thereby the high level of Ig production, supporting humoral immune response (Majid and Andleeb 2019).

To validate the designed vaccine in the screening of immune reactivity by serological test, it is necessary to express the construct in a preferred host like *E. coli* K12 for recombinant proteins. Codon optimization was performed to achieve a high expression designed vaccine in *E. coli* 12. Codon adaptability index (CAI = 1.0) and GC content (51.66%) of the vaccine construct were optimum for high

expression of recombinant protein in the host. The immediate step, hereafter is to express the protein in a preferred host and validate results obtained in this report by performing several immunological assays.

Different variants of SARS-CoV2 were emerged in mean time evading neutralizing antibody response (Edara et al. 2021; Planas et al. 2021). Interestingly, the predicted epitopes do not fall in the region reported as a unique part of different variants of SARS-CoV2 including delta variant (P681R) (UniprotKB. 2021). A vaccine made of such epitopes may work against variants of SARS-CoV2.

Conclusion

With an inadequate number of drugs and the time-consuming process of drug development, the multiplication chain of SARS-CoV2 infection can be controlled only by the potential vaccine. We utilized tools and techniques of immune informatics to design a potential vaccine candidate. This vaccine has epitopes from the S protein of the SARS-CoV2 virus for T and B cell receptors. One more time, This scientific report showed that the bioinformatics approach could effectively be used to develop vaccines in a short time and with incurred less cost. Although in silico results point out the effectiveness of the vaccine, efficacy needs to be analyzed by performing laboratory experiments and animal model studies.

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Data Availability Available on request.

Declarations

Conflict of Interest G H Kathwate declares no conflict of interest.

Animal Rights This article does not contain any studies with human participants performed by the author.

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